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Maternal germline mosaicism of kinesin family member 21A (*KIF21A)* mutation causes complex phenotypes in a Chinese family with congenital fibrosis of the extraocular muscles

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Purpose: To identify the causative mutation with its possible origin in a Chinese family with congenital fibrosis of extraocular muscles type 1 (CFEOM1) and to characterize the ocular phenotypes and lesions in the corresponding intracranial nerves.

Methods: Three affected siblings and their asymptomatic parents underwent comprehensive ophthalmic examinations and neuropathologic analysis involving magnetic resonance imaging (MRI). *KIF21A*, *PHOX2A*, and *TUBB3* genes were sequenced on the leukocyte-derived DNA to detect variants. The disease-linked haplotype was analyzed using four microsatellite markers across the *KIF21A* locus.

Results: All three affected individuals displayed typical CFEOM1. MRI revealed complicated but consistent neuromuscular abnormalities in the two patients examined, including hypoplastic oculomotor nerves, complete absence of bilateral superior rectus muscles, and unilateral absence of the abducens nerve with marked atrophy of the corresponding lateral rectus muscle. A heterozygous hotspot mutation *KIF21A* c.2860C>T was identified in all patients, but it was absent in both parents. Haplotype analysis of the disease locus showed the likely maternal inheritance of the disease-associated haplotype to all three affected offspring, strongly suggesting maternal germline mosaicism of the mutation.

Conclusions: Germline mosaicism of *KIF21A* c.2860C>T is likely to cause the high occurrence of this mutation in the population. This information may be useful for genetic counseling. *KIF21A* mutations can affect the abducens nerve and cause complete absence of the bilateral superior rectus muscles. MRI characterization of new CFEOM1 phenotypes would assist clinical management.

Congenital fibrosis of extraocular muscles (CFEOM) is a group of neurologic maldevelopmental disorders that primarily involve the oculomotor nerves and nuclei or the trochlear nerves and nuclei or both with secondary abnormalities in the correspondingly innervated muscles [1]. CFEOM can be clinically classified into four major categories and several respective subtypes according to its genetic components. Of these, CFEOM type 1 (CFEOM1; MIM 135700) and CFEOM type 3 (CFEOM3; MIM 600638; MIM 609384) can be inherited in an autosomal dominant fashion.

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The diagnosis of CFEOM is usually supported by clinical and genetic findings. CFEOM1, known as the classic form of CFEOM, is typified by congenital non-progressive bilateral external ophthalmoplegia manifesting restricted vertical and horizontal ocular motility and ptosis leading to droopy eyelids and the chin-up position of the head [2]. These phenotypes were generally believed to be the consequences of dysplasia of the oculomotor nucleus and nerve and its innervated muscles (superior, medial, and inferior rectus, inferior oblique, and levator palpebrae superioris) [3]. Magnetic resonance imaging (MRI) has become a routine means of clearly showing the functional anatomy of associated cranial nerves and nuclei and extraocular muscles (EOMs) in the orbits of patients with CFEOM [4]. For patients with CFEOM1, the atrophic levator palpebrae superioris and other EOMs as well as the hypoplastic oculomotor nerves can be detected with MRI.

To date, five loci have been associated with CFEOM, including the *FEOM1* locus [5], the paired-like homeobox 2a (*PHOX2A*) locus [6], the *FEOM3* locus [7], the *FEOM4* locus [8], and the Tukel syndrome gene (*TUKLS)* locus [9]. Among them, three genes were identified. These include *KIF21A* (MIM 608283) [5], mutations in which account for the majority of CFEOM1 and a small fraction of CFEOM3 (CFEOM3A; MIM 600638), the *PHOX2A* gene (MIM 602753) [6], and the *TUBB3* gene (MIM 602661) [7]. Recently, the *TUBB2B* E421K substitution was also identified as a cause of CFEOM [10]. Among the mutations identified in *KIF21A*, the p.R954W mutation is a hotspot, accounting for about 72% to 75% of patients with CFEOM1 [5,11]. However, the mechanism underlying the high frequency of this mutation is poorly understood.

A Chinese family with complex phenotypes of CFEOM1 is discussed. All three siblings are affected and found to carry a heterozygous hotspot mutation of *KIF21A* gene, p.R954W, which is absent from both asymptomatic parents. Haplotype analysis denoted an inheritance mode of maternal germline mosaicism. The phenotypic consistency among all patients strongly suggests that unilateral absence of the abducens is a new trait related to *KIF21A* gene mutation.

METHODS

Family recruitment and clinical evaluations: Institutional ethical approval was granted for the present study, which was conducted in adherence to the tenets of the Declaration of Helsinki. Informed consent was obtained from all participants or their legal guardians throughout the study.

This family was first referred to Tongren Eye Center (Beijing, China) for severely fixed strabismus and ptosis presented in three siblings (Figure 1A). All five available family members, including all three affected siblings and their asymptomatic parents, underwent comprehensive ophthalmic examinations, including routine ophthalmic examinations, strabismus tests, and multipositional high-resolution MRI with a General Electric 1.5-T Twinspeed scanner (GE Healthcare, Chalfont St. Giles, UK). The grading of ptosis was measured using four measurements, including interpalpebral fissure height, marginal reflex distance (MRD1), levator function, and upper lid crease position. Protocols for each measurement have been described previously [12]. Classifications of MRD1 and levator function are detailed in Table 1. Extraocular muscles and intraorbital motor nerve branches were displayed on a 2-mm-thick T1-weighted image in triplanar scans with dual-phased coils. Motor nerves were seen in the cistern on a General Electric 3D FIESTA (0.6 mm thick) MRI scanner (GE Healthcare) with head coils.

Mutation screening and sequence analysis: Each participant donated a 5 ml venous blood sample for genomic DNA extraction. Ethylene diamine tetraacetic acid (EDTA) treated tubes were used for blood collection. Genomic DNA was isolated using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) per the manufacturer's protocol. Genomic DNA samples were preserved at -20 °C. Exons and flanking exon-intron boundaries of *KIF21A* (38 exons), *PHOX2A* (three exons), and *TUBB3* (five exons) were amplified in all collected samples (three patients and their parents) with polymerase chain reaction (PCR). Reference sequences for *KIF21A*, *PHOX2A*, and *TUBB3* were ENST00000361961, ENST00000298231, and ENST00000556922, respectively, and were taken from the ENSEMBL Human Genome Browser Map. The PCR amplicons were subsequently purified, sequenced in both directions, and analyzed according to methods previously described [13].

Haplotype analysis: Four microsatellite markers flanking the *KIF21A* gene, including D12S1668, D12S1048, D12S2194, and D12S331, were selected for haplotype analysis (Figure 1A) using genomic DNA obtained from all available family members. Fluorescent-labeled primers were designed using the uniSTS database. Family and haplotype data were generated using Cyrillic (version 2.1) software and confirmed by inspection.

Conservation analysis: To confirm whether these mutated amino acids are evolutionarily conserved, we aligned the orthologous protein sequences of the KIF21A genes of the following species using Vector NTI Advance 11 software (Invitrogen, Grand Island, NY): *Homo sapiens*, *Gorilla gorilla*, *Canis lupus familiaris*, *Bos taurus*, *Sus scrofa*, *Rattus norvegicus*, *Gallus gallus*, and *Drosophila melanogaster.*

RESULTS

Clinical findings: Family and personal histories were carefully reviewed. All three affected siblings were born after uneventful full-term pregnancies and deliveries, and no other family members were reported to have ophthalmic conditions. Ophthalmic evaluations revealed CFEOM1 phenotypes in all three affected individuals (Table 2), but nothing remarkable was observed in the parents. All three patients displayed severe bilateral ptosis with absence of Bell's phenomenon, compensatory chin elevations, and fixed strabismus in the hypotropia position (varying from 20 to 25 prism diopters [PD]) and the esotropia position (varying from 35 to 45 PD; Table 2 and Figure 2). Severely impaired vertical and horizontal ocular motility was observed in all three patients (Table 2). Synergistic convergence when attempting upgaze was observed in all three affected siblings. In addition,

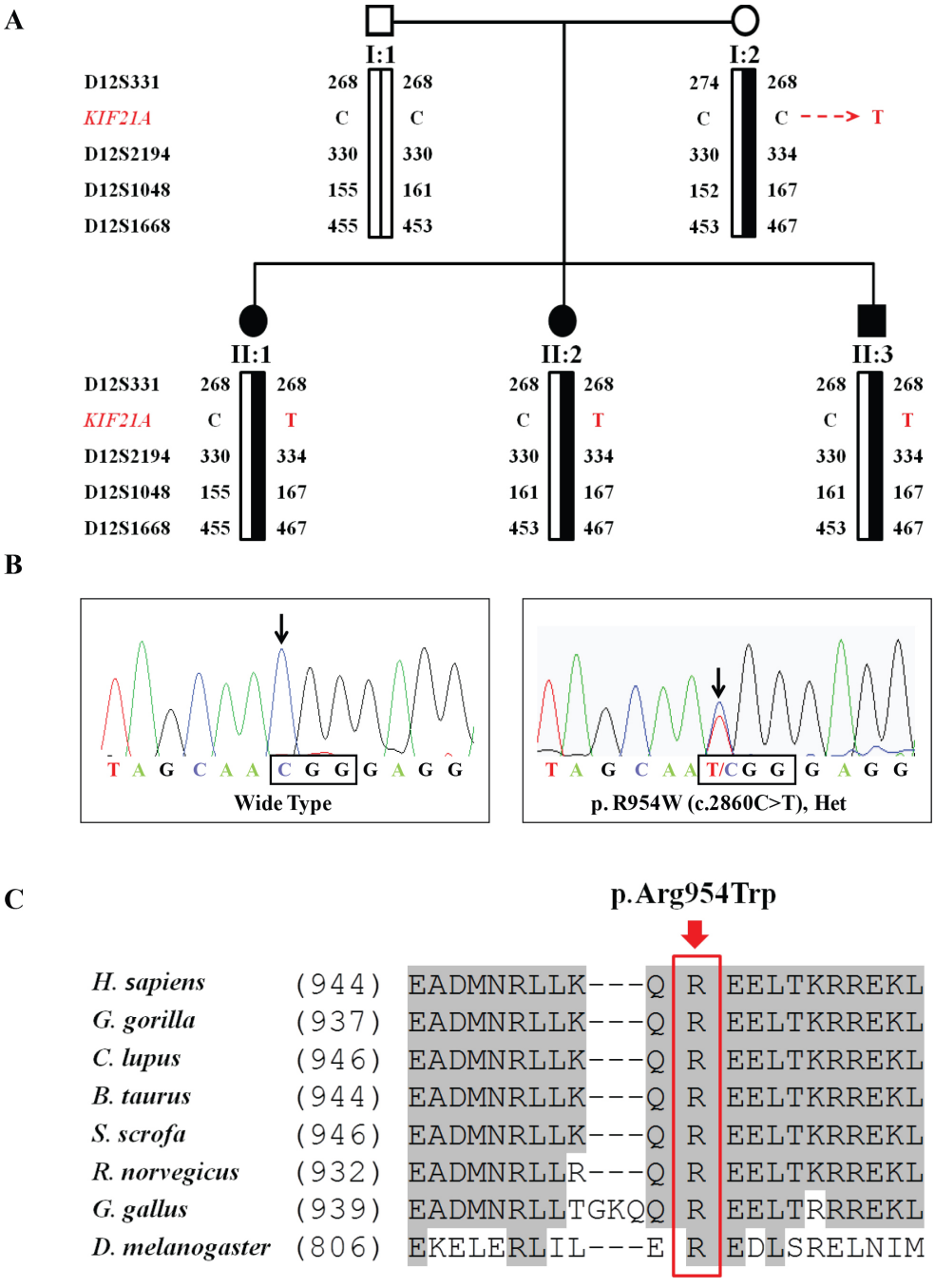

Figure 1. Pedigree and genetic analysis of the family with congenital fibrosis of extraocular muscles. **A**: Family pedigree shows that all three siblings have congenital fibrosis of extraocular muscles (CFEOM) while both parents are asymptomatic. Haplotype analyses using the four microsatellite markers (D12S331, D12S1048, D12S1668, and D12S2194) flanking the kinesin family member 21A (*KIF21A*) gene denote the affected haplotype (black bar) shared by all patients and their mother. Genotyping data for each marker involved the size of the products of the polymerase chain reaction (PCR). The dashed arrow indicates possible maternal germline mosaicism. Black filled symbols and blank symbols represent affected and unaffected status, respectively. **B**: The DNA chromatogram sequence for the *KIF21A* gene shows the three affected siblings are heterozygous for the c.2860G>T mutation, and their parents were homozygous for the wild-type allele. The arrow points to the site of the base substitution. **C**: The missense mutation *KIF21A* p.R954W was evolutionarily conserved among various species.

forced duction testing demonstrated marked restrictions in passive elevation of the globes in all three patients. No remarkable findings were detected in the anterior segments or during fundus examinations. Patient II:1 underwent bilateral frontalis sling surgery at age 2 and bilateral inferior rectus recession surgery at age 6. Patient II:2 had bilateral inferior rectus recession surgery at age 4. These operations slightly improved the patients' hypotropia at the primary position and compensatory head position. However, as expected, no improvement in ocular motility was observed in the two patients after any of these surgeries.

Orbital imaging: In addition to the characteristic CFEOM1 phenotypes described above, severe abnormalities of the cranial nerves and their innervated EOMs were also identified in the two affected siblings examined (Figure 3 and Figure 4). On MRI, hypoplastic oculomotor nerves with complete absence of bilateral superior rectus (SR) muscles,

Table 1. Classifications of MRD1 and levator function

MRD1
Normal (+ 4.0 mm ~ + 4.5 mm)
Mild Ptosis (+ 1.5 mm)
Moderate Ptosis (+ 0.5 mm)
Severe Ptosis (- 0.5 mm)
Levator Function
Excellent (13 mm ~ 15 mm)
Good (8 mm ~ 12 mm)
Fair (5 mm ~ 7 mm)
Poor (≤4 mm)

Abbreviation: MRD1: marginal reflex distance.

hypoplastic medial rectus (MR) muscles, and inferior rectus (IR) muscles were observed in continuous sections in both examined patients at the primary position. Interestingly, in the more affected side (left side) of the oculomotor nerves in the two patients, unilateral absence of abducens nerves with remarkable atrophy of the innervated LR muscle was observed (Figure 3). Consistently, the two patients were unable to abduct or adduct their left eyes, but they could abduct their right eyes relatively normally (Table 2 and Figure 2). Although patient II:3 was too young to undergo MRI, he presented similar eye motility (Table 2), indicating that he also had abducens nerve atrophy of the left eye.

Identification of mutation and haplotype analysis: Genetic analysis demonstrated a heterozygous recurrent mutation *KIF21A* c.2860C>T carried by all three patients but absent in the parents (Figure 1A,B). Because all three siblings carried this mutation, these mutations were not likely to be random de novo mutations but were rather transmitted via parental germline mosaicism [14]. To test this idea, it was first determined whether the mutations had originated in the patients' mother or father. Haplotype analysis performed using four microsatellite markers surrounding *KIF21A* defined a disease-associated haplotype shared by all three affected siblings and the asymptomatic mother (Figure 1A), strongly suggesting a maternal origin of the germline mosaicism for c.2860C>T mutation (Figure 1A).

Bioinformatic analysis: The *KIF21A* protein comprised a kinesin-motor domain, coiled-coil regions, alternatively

Table 2. Ophthalmic investigations in three affected members

Age (year) / Sex		II:1 8 / F	II:2 5 / F	II:3 2 / M
BCVAs (log MAR) (O.D. / O.S.)		0.8 / 0.8	0.4 / 0.3	NA / NA†
Refractive Errors	**O.D.**	-1.00S+2.50C*70	-2.50S+4.00C*105	+2.50S+2.50C*90
	O.S.	-1.50S+2.00C*80	-3.00s+4.50C*95	+3.00S+2.75C*85
Pupils		NOR	NOR	NOR
MRD1 (O.D. / O.S.)		Severe / Severe	Severe / Severe	Severe / Severe
Levator Function (O.D. / O.S.)		Poor (3 mm) / Poor (4 mm)	Poor (2 mm) / Poor (2 mm)	Poor (2 mm) / Poor (2 mm)
Strabismus	**O.D.**	25 PD of Hypo; 35 PD of Eso	25 PD of Hypo; 45 PD of Eso	25 PD of Hypo; 35 PD of Eso
	O.S.	20 PD of Hypo; 35 PD of Eso	25 PD of Hypo; 45 PD of Eso	25 PD of Hypo; 35 PD of Eso
Ocular Motility				
O.D.	***Hyperduction***	Fixed	Fixed	Fixed
	Hypoduction	Fixed	Fixed	Fixed
	Abduction	NOR	NOR	NOR
	Adduction	Limited	Limited	Fixed
O.S.	***Hyperduction***	Fixed	Fixed	Fixed
	Hypoduction	Fixed	Fixed	Fixed
	Abduction	Fixed	Fixed	Fixed
	Adduction	Fixed	Fixed	Fixed
Compensatory Head Position		Yes	Yes	Yes

† Patient II:3 was only 2-year-old at the time of examination, which was too young to cooperate with the examination of best corrected visual acuity. Thus, his BCVAs were not attainable. **Abbreviations:** F, female; M, male; BCVA, best-corrected visual acuity; O.D., right eye; O.S., left eye; NA, not available; NOR, normal; PD, prism diopters; Hypo, hypotropia; Eso, esotropia; Hyperd, hyperduction; Hypod, hypoduction; Abd, Abduction; Add, Adduction; CHP, compensatory head position.

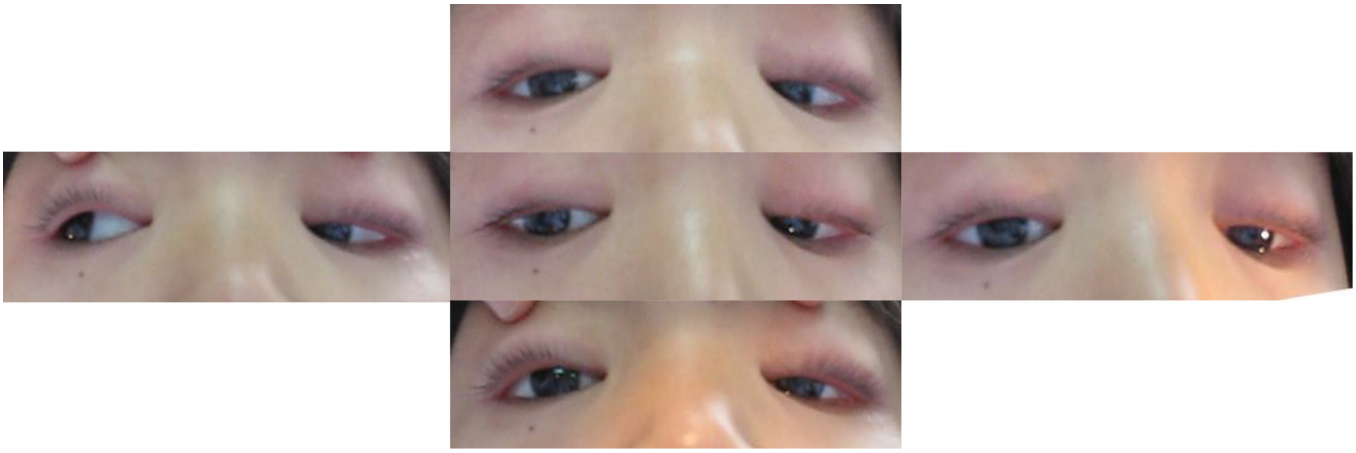

Figure 2. Strabismus test of patient II:2 at five diagnostic positions. At the primary position, the right eye was fixed at the hypotropia (HT) position, and the left eye was fixed at the esotropia (ET) and HT positions. Minimal vertical motilities and adductions were noticed in both eyes. Notably, the right eye showed nearly normal abduction, and the left showed none. This patient had undergone bilateral inferior rectus recession surgery at age 4.

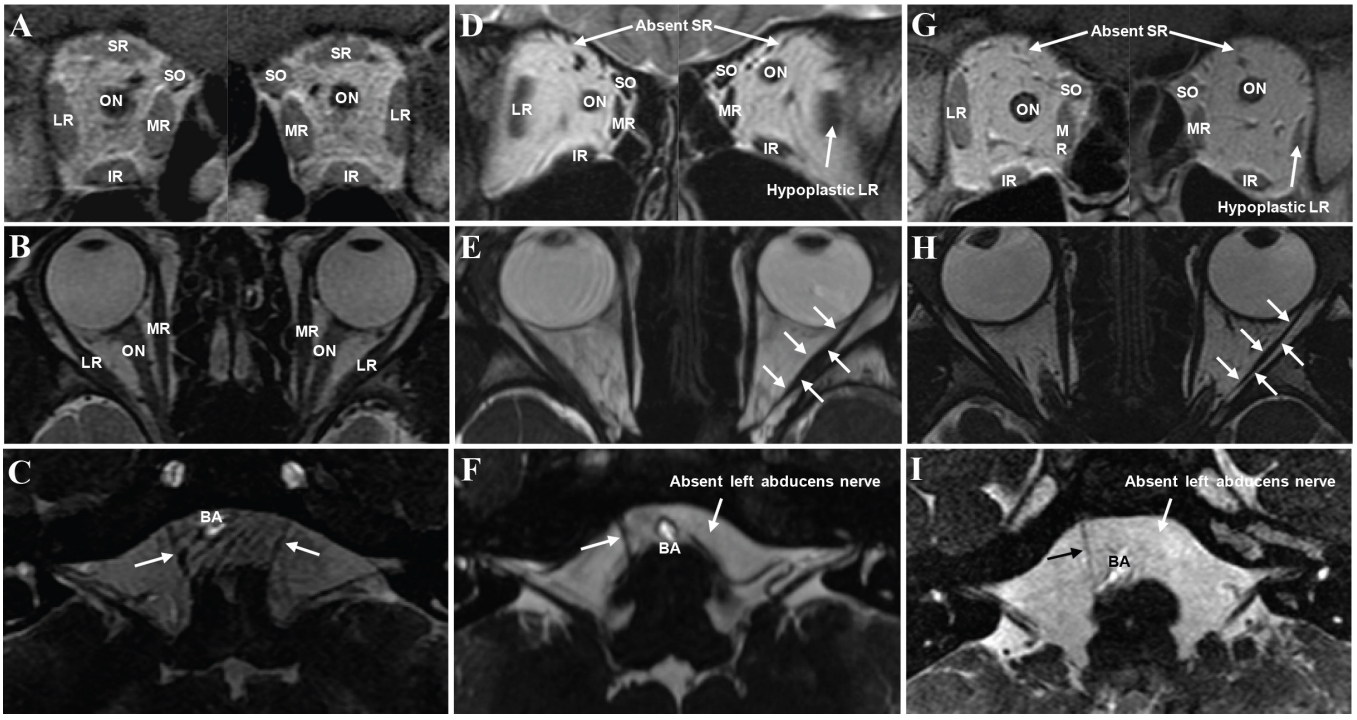

Figure 3. Imaging of the extraocular muscles and abducens nerves. **A**–**C**: Normal aspects of the extraocular muscles (EOMs) and bilateral abducens nerves are presented by coronal and axial magnetic resonance imaging (MRI) in a control individual. **D**: Coronal MRI of the bilateral orbits of patient II:1 showed the absence of bilateral superior rectus (SR) muscles and hypoplasia of the left lateral rectus (LR) muscle but a normal LR muscle of the right eye. **E**: The axial MRI of patient II:1 showed adduction of the left eye. The LR muscle (arrows) of the left eye was small and had a string-like configuration, suggesting fibrosis. In contrast, the LR muscle of the right eye was of normal size with a spindle shape. **F**: The axial MRI illustrates the absence of the left abducens nerve in patient II:1. **G**–**I**: Patient II:2 presented similarly to patient II:1 by showing absence of bilateral SR muscles and small left LR muscle in the coronal MRI. The axial MRI of both orbits reveals the fibrosis of the left LR muscle (arrows) and adduction of left eye. An absence of the left abducens nerve was also demonstrated by the axial MRI. Abbreviation: MR, medial rectus; IR, inferior rectus; SO, superior oblique; ON, optic nerve; BA, basilar artery.

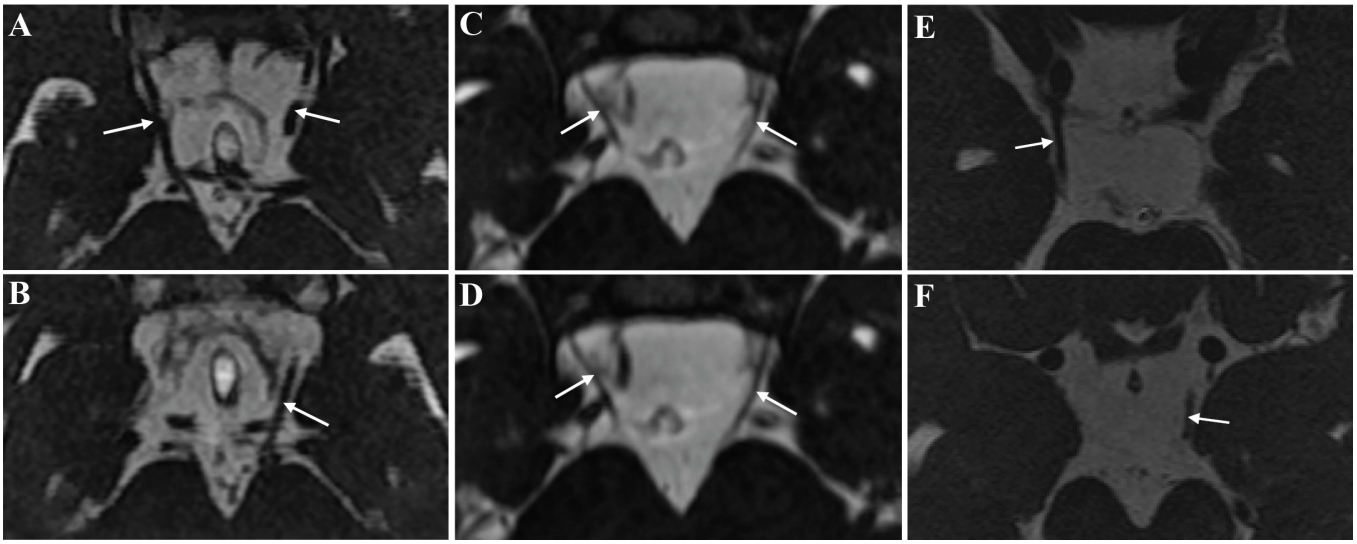

Figure 4. Imaging of the oculomotor nerves. **A**–**B**: Bilateral oculomotor nerves demonstrated by the axial magnetic resonance imaging (MRI) of a normal control. **C**–**D**: The axial MRI indicates bilateral hypoplastic oculomotor nerves in patient II:1. **E**–**F**: Patient II:2 presented hypoplasia similar to that of patient II:1, according to the axial MRI.

spliced regions, and seven WD repeats in the tail. Among all the regions, the c.2860C>T mutation, which resulted in replacement of a hydrophilic amino acid (arginine) with a hydrophobic amino acid (tryptophan), is located at position a of a heptad (a–g) repeat in the second coiled-coil region of the *KIF21A* stalk [5]. Analysis using Vector NTI Advance 2011 software showed the arginine 954 residue was highly conserved among orthologous proteins from eight species (Figure 1C).

DISCUSSION

The diversity of the clinical findings regarding the causes of blepharoptosis and strabismus makes it difficult for ophthalmologists to give specific diagnoses. Reportedly, genetic investigation has become a useful and complementary tool in assisting clinical and prenatal diagnosis. Therefore, it is of significant importance to carefully characterize the complex phenotypes and endophenotypes for better understanding of the causes of this disease, allowing doctors to make more reliable clinical diagnoses.

The CFEOM phenotype is usually correlated with abnormities in oculomotor nerves and trochlear nerves. Absence of the abducens nerves has only rarely been reported in patients with CFEOM [3], and cosegregation with *KIF21A* mutations in familial cases is rare. To date, 12 missense mutations and one deletion in the *KIF21A* gene have been identified in patients presenting with CFEOM1 [2,5,15-17]. Among these, *KIF21A* c.2860C>T has been identified as the most common mutation worldwide, with a prevalence between 72% and 75% [5,11]. This is the first report to show that the hotspot mutation *KIF21A* c.2860C>T correlates with a panel of new traits, including the absence of the bilateral SR muscles, the absence of the left abducens nerve, and atrophy of the left LR muscle as demonstrated by MRI. Consistent with the MRI findings, all three patients showed much better abduction of the right eye than of the left. Patient II:3 was too young for a coordinated MRI examination, but motility tests indicated that he also has left abducens nerve hypoplasia. These data may exclude the possibility that those new traits are acquired dispositions based on the young age of the two patients when MRI was performed.

Mosaicism in germ cells has been recognized as a significant mechanism involved in the origin of genetic disorders. Theoretically, based on the timing of postzygotic mutations, the generation of such mosaicism can be divided into two major groups: germline mosaicism, which occurs as a germ cell continues to divide, and somatic mosaicism, which occurs in a somatic cell before separation into germinal cells, presenting in somatic and germinal cells [14,18]. Thus far, germline mosaicism has been witnessed in a range of diseases, including Duchenne muscular dystrophy (DMD) [19] and osteogenesis imperfecta [20]. A previous study showed that 80% of retinoblastoma are due to de novo mutations, making retinoblastoma the most common ophthalmic disease caused by germinal mosaicism [21]. Some syndromes with eye involvement have also been reported to be associated with germline mosaicism. These include Lowe syndrome [22], incontinentia pigmenti (IP) [23], Marfan syndrome [24],

sex determining region Y-box 2 (SOX2)-related eye disorders [25], and ankyloblepharon-ectodermal defects-cleft lip/palate (AEC) syndrome [26]. Germline mosaicism has been suggested as an inheritance mode for CFEOM1. However, there is a lack of conclusive evidence [27,28].

In the family under examination, the fact that all three siblings had the *KIF21A* mutation in leukocyte-derived DNA and both parents did not indicated that the mutation was unlikely to be random de novo mutation but rather transmitted via parental germline mosaicism [14]. To test this idea, haplotype analysis was used to determine from which parent the mutation originated. The fact that the disease-associated maternal allele was shared by all three patients strongly supports an inheritance mode of maternal germline mosaicism in the family. Because germ cells from females (eggs) are difficult to access [14], it is usually challenging to confirm such maternal germline mosaicism, and this was certainly true of the present case. Maternal germline mosaicism has been identified in another Chinese family with CFEOM1. This second family is from a different part of China [28]. The disease-associated maternal allele is shared by both siblings, which is consistent with the present findings and indicates the great harm caused by maternal germline mosaicism. Genetic consultations for such families are quite important and should be emphasized. Further investigations into the etiology and pathogenesis of CFEOM1 with maternal germline mosaicism are needed.

KIF21A c.C2860 is located in a methylated CpG (mCpG) dinucleotide [29]. The mCpG sequences are mutational hotspots in human genetic diseases. Random deamination of 5-methylcytosines causes these mutations [30], indicating that the methylation status of the mutated sites may be taken as an indicator when judging the possibility of potential mutations. Germline mosaicism for a C→T nucleotide substitution, as in the present case, can often be caused by methylation on the CpG dinucleotide. Taken together, these findings point to the possibility that the CpG dinucleotide at the hotspot site is highly methylated. The CpG nucleotide could be more mutable in germ cells than in other types of cells. This theory provides a rationale for using the methylation status of frequently mutated CpG sites in the leukocyte-derived genomic DNA as molecular markers in the genetic counseling of such families.

Since men produce more germ cells than women, the odds of transmission of a given mutation from paternal germline mosaicism differs from that of maternal mosaicism [18]. Previous reports of a family with CFEOM1 with possible paternal germline mosaicism of the hotspot mutation showed that 33% of the offspring of asymptomatic parents are affected [27], but in the present study, 100% of the offspring were affected. At this point, the present findings indicate that maternal germline mosaicism has a more frequent impact on offspring regarding the transmission of CFEOM1.

In conclusion, a novel panel of traits for the *KIF21A* c.2860C>T mutation as examined with MRI is reported. These traits include bilateral hypoplastic oculomotor nerves with the complete absence of bilateral SR muscles and the absence of the unilateral abducens nerve with marked atrophy of the corresponding LR muscle, suggesting a novel genotype-phenotype correlation. This is the first report to confirm maternal germline mosaicism transmission for the present mutation, which is also correlated with increased likelihood of transmission to offspring. Mutation screening of genomic DNA derived from germ cells should be considered in the genetic counseling of families with conditions with de novo like modes of inheritance.

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